Contributions of Glycoprotein Ib and the Seven Transmembrane Domain Receptor to Increases in Platelet Cytoplasmic [Ca^{2+}] Induced by α-Thrombin

Nicholas J. Greco,*,‡ Narendra N. Tandon,‡ Glen D. Jones,‡ Robyn Kornhauser,‡ Barrington Jackson,‡ Naomasa Yamamoto,§ Kenjiro Tanoue,§ and G. A. Jamieson‡

Platelet Biology Department, American Red Cross, Rockville, Maryland 20855, and Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

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ABSTRACT: The individual contributions of glycoprotein Ib (GPIb) and the seven transmembrane domain receptor (STDR) to increases in platelet $[Ca^{2+}]_i$ induced by α-thrombin or the tethered ligand peptide (TLP; SFLLRNPNDKYEPF) have been determined in control platelets, in platelets where the thrombin binding site on GPIb was blocked with the monoclonal antibodies TM60 and LJ-Ib10, in platelets where access of thrombin to the STDR was blocked by polyclonal antipeptide antibodies, and in Bernard-Soulier platelets which constitutively lack GPIb. Curve-fitting analyses (LIGAND) showed that binding of PPACK-thrombin and α-thrombin to the moderate-affinity site was not detected in the best-fit model in the presence of anti-STDR antibodies although with α-thrombin there was also decreased binding at the high-affinity site. Conversely, TM60 blocked binding of α-thrombin to the high-affinity site but also decreased binding at the moderate affinity site. Separately, either TM60 or anti-TNA (150 μg/mL) reduced thrombin (0.5 nM)-induced elevations in $[Ca^{2+}]_i$ to 50% of control values, but Ca^{2+} elevations were essentially abrogated (4.2 ± 5%) when the two were added in combination. $[Ca^{2+}]_i$ dose-response curves for α-thrombin were curvilinear and were only 50% of controls in the presence of anti-GPIb or anti-STDR antibodies at up to 10 nM α-thrombin, with their greatest sensitivity being below 2 nM. With Bernard-Soulier platelets, changes in $[Ca^{2+}]_i$ were not detectable at ≤0.5 nM α-thrombin but were also 50% of controls at 5−10 nM α-thrombin. $[Ca^{2+}]_i$ responses to TLP (1−100 μM) of antibody-blocked platelets were identical to those of controls whereas responses were ∼50% of controls in Bernard-Soulier platelets. The rate of increase in $[Ca^{2+}]_i$ in controls was twice that seen in antibody-blocked platelets and about 5-fold greater than in Bernard-Soulier platelets. These results demonstrate that *both* GPIb and the STDR are required to ensure the optimal rate and extent of platelet activation over a range of α-thrombin concentrations (0.3−10 nM) and that the STDR corresponds to the previously described moderate-affinity thrombin receptor.

Thrombin is a potent cell agonist, but the nature of the receptor, or receptors, by which it induces activation of platelets and other cells has been a matter of controversy [for reviews, see Jamieson (1988) and Greco and Jamieson (1991)].

The cDNA encoding a thrombin receptor present in platelets and vascular endothelial cells has recently been isolated, and the deduced amino acid sequence has shown it to be a member of the seven transmembrane domain receptor (STDR)[1] family (Vu et al., 1991; Rasmussen et al., 1991). This receptor is thought to bind α-thrombin through a domain (EPFWEDEEKNES) having homology to the thrombin binding domain DFEEIPEE of hirudin. Binding to this site allows proteolytic cleavage at a DPR/SFLL sequence in the STDR, leading to a new amino terminus which acts as a tethered ligand interacting with the receptor itself to effect activation. These results have led to wide acceptance of the significant role of this receptor in platelet activation and to the assumption that it can explain all of the effects of α-thrombin on platelets.

There is, however, extensive evidence that there are two different types of thrombin receptors on platelets (Yamamoto et al., 1991). These receptors differ in their sensitivity to chymotrypsin (McGowan & Detwiler, 1986) and *Serratia* protease (Yamamoto et al., 1991), in their requirements for receptor occupancy (Holmsen et al., 1981; Huang & Detwiler, 1987), and in the roles of guanine nucleotide regulatory proteins (G proteins) (Houslay et al., 1986; Grandt et al., 1986) and Na^+ ions (Sweatt et al., 1986). Moreover, two types of binding site can be detected in the physiologically relevant range of thrombin concentrations—a high-affinity site with a K_d of ∼0.5 nM (∼50 sites/platelet) and a moderate-affinity site of K_d ∼50 nM (∼2000 sites/platelet) (Harmon & Jamieson, 1985; De Marco et al., 1991). PPACK-thrombin blocked at its active site binds to these platelet sites similarly to α-thrombin and can completely

* Address correspondence to this author at the American Red Cross, Jerome Holland Laboratory, Platelet Biology Department, 15601 Crabbs Branch Way, Rockville, MD 20855. Phone: (301) 738-0736. FAX: (301) 738-0794. Internet:Greco@USA.red-cross.org.

‡ American Red Cross.

§ Tokyo Metropolitan Institute of Medical Science.

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[1] Abbreviations: STDR, seven transmembrane domain receptor; TLP, tethered ligand peptide SFLLRNPNDKYEPF; TNA peptide, peptide TNATLDPRSFLLRN of the STDR which is thought to interact with the active site of α-thrombin; EPF peptide, peptide EPFWEDEEKNES of the STDR interacting with the exosite of α-thrombin; LLR, peptide LLRNPNDKYEPF(C) bridging the TNA and EPF peptides of the STDR; DEG peptide, peptide DEGDTDLYDYYPEEDTEGD representing the thrombin binding domain of GPIbα; FITC, fluorescein isothiocyanate; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone.

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abrogate thrombin-induced platelet responses, demonstrating that these are not merely binding sites but are receptors coupled to platelet activation (Greco et al., 1995).

It has been well established that the high-affinity α-thrombin binding site of platelets is located on GPIbα [for review, see Jamieson (1988) and Greco and Jamieson (1991)]. Evidence that GPIb acts as a functional receptor for α-thrombin and not merely as a binding site was obtained by the finding that five different monoclonal antibodies against the thrombin binding domain in GPIbα were able to inhibit thrombin-induced platelet activation (Kunicki et al., 1983; Mazurov et al., 1991; McGregor et al., 1983; Yamamoto et al., 1985; De Marco et al., 1991) while more extensive studies with three of these antibodies showed that they were also able to inhibit the high-affinity binding of α-thrombin to platelets (Yamamoto et al., 1991; Mazurov et al., 1991; De Marco et al., 1991).

The GPIb complex comprises GPIbα, GPIbβ, and GPIX, it has a molecular mass of ∼190 kDa, and there are approximately 30 000 copies present on the platelet surface. However, the form of GPIb that constitutes the high-affinity thrombin binding site (50 copies) appears to be present as a multimolecular complex of membrane components since it has a functional molecular size of ∼900 kDa as determined by radiation inactivation and target analysis (Harmon & Jamieson, 1985), a technique which gives the sum of the molecular weights of the interacting components required to elicit high-affinity binding (Fraser & Venter, 1987): it is convenient to use the term "supercomplex" to distinguish this complex from the GPIb/IX complex itself. There is other evidence for supercomplexed forms of GPIb: thrombin-induced receptor oligomerization may have a role (Ganguly & Gould, 1979), and GPV is known to physically associate with the GPIb/IX complex which may exist as $(\alpha_2\beta_2\gamma_2\delta)_\eta$ where α, β, γ, and δ represent the polypeptides GPIbα, GPIbβ, GPIX, and GPV, respectively, and η indicates the association of multiple complexes (Lopez, 1994). Furthermore, the phospholipase A_2-related signal transduction molecule 14-3-3 is associated with GPIb and is coimmunoprecipitated by anti-GPIb antibodies (Du et al., 1994). This interpretation of supercomplexed forms of GPIb is supported by electron microscopy studies showing that the GPIb/IX complex exists in clusters on the membranes of unactivated platelets (Polley et al., 1981) and by rapid freeze-etching of platelets which shows clusters of GPIb/IX molecules at the branch points of assembled actin binding proteins (Hartwig & DeSisto, 1991).

All the components of the GPIb/IX complex itself have been cloned (Lopez, 1994), but the mechanism by which the binding of α-thrombin to GPIb initiates platelet activation through the high-affinity pathway is not known, and its significance as a thrombin receptor has not been widely accepted.

In the present study, we have evaluated the effects of antibodies to the STDR and GPIb on thrombin binding and changes in $[Ca^{2+}]_i$ induced by α-thrombin and the activating tethered ligand peptide (TLP) of the STDR using three different types of platelet preparations: these are (a) platelets in which access to the high-affinity thrombin binding domain on GPIbα has been blocked by the monoclonal antibodies TM60 or LJ-Ib10; (b) platelets in which access to the STDR was blocked with polyclonal antipeptide antibodies against the binding domain of that receptor; and (c) Bernard-Soulier platelets which constitutively lack GPIb.

EXPERIMENTAL METHODS

Fura-2/AM was obtained from Molecular Probes (Eugene, OR). Highly purified human α-thrombin (activity ≥3000 units/mg) was obtained from Sigma or purified from plasma in this laboratory (Greco et al., 1990); at this level of purity, 0.1 unit/mL α-thrombin equals 1 nM. PPACK-thrombin was prepared as described (Greco et al., 1990). The monoclonal antibodies used were TM60 (Yamamoto et al., 1985) and LJ-Ib10 (De Marco et al., 1991), both directed against the thrombin binding domain of GPIbα, and ATAP138 against the STDR (Brass et al., 1992). FITC and FITC-conjugated goat anti-mouse and goat anti-rabbit antisera were obtained from Sigma. S2238 (200 μM) hydrolysis was measured as described (Greco et al., 1990). One-tailed *p* values were calculated by paired *t*-tests.

The following peptides were synthesized on a MilliGen Model 9050 peptide synthesizer and purified by HPLC on a C-18 μBondapak column (Waters, Bedford, MA): (a) the tethered ligand peptide SFLLRNPNDKYEPFC-NH_2, corresponding to the sequence of the tethered ligand; (b) the TNA peptide TNATLDPRSFLLRNP, constituting the domain of the cloned STDR that recognizes the active site of α-thrombin; (c) the EPF peptide EPFWEDEEKNES of the STDR that is postulated to recognize the anion binding exosite of α-thrombin; (d) the LLR peptide LLRNPNDKYEPFC-NH_2 that bridges the TNA and EPF peptides of the STDR; and (e) the DEG peptide DEGDTDLYDYYPEEDTEGD, corresponding to the high-affinity thrombin binding domain of GPIbα.

Antibody Preparation. HPLC-purified peptides (4 mg) were dissolved in 83 mM NaH_2PO_4, 0.9 M NaCl, and 0.1 M EDTA, pH 7.2, and were then coupled to keyhole limpet hemagglutinin (KLH) with 0.15% glutaraldehyde (Harlow & Lane, 1988) while the TLP analogue LLRNPNDKYEPFC-NH_2 was coupled to maleimide-activated KLH (3 h, 22 °C): coupling efficiency was evaluated by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). Following dialysis against phosphate-buffered saline (PBS), KLH-conjugated peptides were stored at −20 °C. Initial immunization in New Zealand White rabbits used 0.5 mg of peptide-KLH conjugates in 0.5 mL of PBS mixed with 0.5 mL of Freund's complete adjuvant. Subsequent boosts were with 0.2 mg of peptide-KLH conjugates and Freund's incomplete adjuvant. Peptide recognition and antibody titers were assayed by ELISA 2 months after the initial injection. Peptides (5 μg) were coated overnight at 4 °C to Immulon or Maxisorb F8 microtiter plates (Dynatech Laboratories, Chantilly, VA) in a 0.1 M $NaHCO_3$, pH 9.5, buffer. Total immunoglobulins, which were isolated using ammonium sulfate precipitation and protein A-Sepharose chromatography (Pierce, Rockford, IL), or serially-diluted heat-inactivated antisera (50 μL) were added to the plates which were then incubated (2 h, 37 °C) before extensive washing. Polyclonal antibodies binding to the peptides were visualized using a goat anti-rabbit alkaline phosphatase-conjugated antibody.

Platelet Preparation. Platelets were isolated from human blood anticoagulated with citrate/phosphate/dextrose/adenine (CPD-A1) obtained from volunteer donors through American Red Cross Blood Services and were used within 3 h of collection. Platelet-rich plasma (PRP) was obtained by centrifuging each unit of whole blood at 1400*g* for 5 min at 22 °C to sediment red cells and then transferring the PRP to the satellite bag.

PGE_1 (1 μg/mL) and citric acid (4.2 mM) were added to the PRP to prevent activation during washing. When $[Ca^{2+}]_i$ measurements were to be carried out, the PRP was at this point incubated with Fura-2/AM (2.5 μM), for 30 min at 37 °C. Platelets were then sedimented by centrifugation at 1200*g* for 10 min and suspended in wash buffer (5.5 mM dextrose, 128 mM NaCl, 4.26 mM Na_2HPO_4, 7.46 mM NaH_2PO_4, 4.77 mM trisodium citrate, and 2.35 mM citric acid, pH 6.5) containing 0.35% bovine serum albumin. Contaminating erythrocytes and white blood cells were removed by further centrifugation at 800*g* for 15 s. The platelets were sedimented by centrifugation at 1200*g* for 10 min and suspended in Tyrode's-HEPES buffer (5.5 mM dextrose, 137 mM NaCl, 2.6 mM KCl, 13.8 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 0.36 mM NaH_2PO_4, and 10 mM HEPES, pH 7.35) supplemented with 0.35% albumin, at a concentration of 1×10^9 platelets/mL. In some experiments, platelets were isolated in the presence of heparin and apyrase (Yamamoto et al., 1991).

Blood anticoagulated with CPD-A1 was obtained from a well-characterized Bernard-Soulier patient (Jamieson & Okumura, 1978) and was allowed to sediment at 1*g* for 5 h. The supernatant PRP ($\sim 10^8$ platelets/mL) was centrifuged briefly (1500*g*, 10 s) to remove contaminating erythrocytes and was then loaded with Fura-2/AM and further processed as described above for normal platelets.

Flow Cytometry. Platelets were washed twice using platelet wash buffer before final suspension at 2×10^8/mL in modified Tyrode's-HEPES buffer with added 2 mM EGTA and 2 mM EDTA (Tyrode's-HEPES/EGTA/EDTA). To determine the binding of antibodies, intact platelets (100 μL) were mixed with 1 μg of primary antibody, and after 15−30 min at 4 °C, 0.5 mL of Tyrode's-HEPES/EGTA/EDTA was added, and platelets were recovered by centrifugation (5 min, 1400*g*). Platelets were washed 1 time with Tyrode's-HEPES/EGTA/EDTA without resuspension and were then resuspended in 100 μL of modified Tyrode's buffer, incubated for 15−30 min at 4 °C with 1 μg of FITC-conjugated goat anti-mouse or goat anti-rabbit secondary antibody, and recovered by centrifugation at 1400*g*. With both the polyclonal and monoclonal preparations, final resuspension was in modified Tyrode's buffer, and flow cytometric analysis was carried out using a Becton-Dickinson FACScan. Data are shown for dot histograms after evaluation of 10 000 events, and comparisons were made using the mean values of the histograms.

With the monoclonal antibodies TM60 (55 μg) and ATAP138 (30 μg), direct labeling was carried out with FITC (1.4 μg) in the dark for 16 h at 4 °C (Harlow & Lane, 1988). To remove unincorporated FITC, ammonium chloride (50 mM) was incubated with the reaction mixture for 2 h prior to chromatography on Sephadex G25 in PBS buffer in the presence of 0.1% xylene cylanol and 5% glycerol. The FITC-conjugated antibody was eluted with phosphate-buffered saline and the 495 nm/280 nm ratio determined. These values were 0.64 and 2.01, respectively, for TM60 and ATAP138.

Measurement of Intracellular Ca^{2+}. Thrombin-induced changes in intracellular Ca^{2+} were monitored using the fluorescent probe Fura-2 as previously described (Greco et al., 1990). Antibodies were incubated with Fura-2-loaded platelets for 10−15 min at room temperature and then for 1 min at 37 °C prior to the addition of 1 mM $CaCl_2$; α-thrombin or peptide was then added within 60 s. Changes in fluorescence were monitored in a spectrofluorometer (MPF-44B; Perkin Elmer, Norwalk, CT) set at excitation and emission wavelengths of 339 and 500 nm, respectively, and slit widths of 6 nm or in a Perkin-Elmer LS50B at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm (slit widths, 10 nm). Calibration of the intracellular Ca^{2+} concentrations was separately determined for each platelet preparation by addition of digitonin (50 μM) to completely lyse the platelets and adding either $CaCl_2$ (1 mM) or EGTA (12 mM) to determine the maximum and minimum levels of fluorescence (*F*); all signals were corrected for extracellular Fura-2, and the K_d for Fura-2 was taken as 224 nM in the calculation of intracellular Ca^{2+} concentration (Pollock et al., 1986).

Thrombin Binding. α-Thrombin and PPACK-thrombin were iodinated with Iodogen as previously described (Harmon & Jamieson, 1985) and separated from free iodine on a PD10 column (Sephadex G25M) equilibrated with 0.2 M NaCl, 10 mM NaH_2PO_4, pH 7.0, yielding a product containing >90% trichloroacetic acid-precipitable radioactivity. Competition binding isotherms were determined with a tracer concentration of ^{125}I-α-thrombin or ^{125}I-PPACK-thrombin (0.1 nM) and increasing concentrations of the nonlabeled ligands up to 1 μM final concentration in Tyrode's-HEPES buffer, pH 7.4, with added PEG 6000 (0.6%) and bovine serum albumin (1%). The ratio of bound to total ligand was maintained at ≤0.1, and bound and free ligands were separated by centrifugation (Harmon & Jamieson, 1985). Assays were carried out in triplicate at 20−22 different α-thrombin concentrations (Munson, 1983). In evaluating the inhibitory effects of the antibodies, washed platelets (1×10^9 platelets/mL) were preincubated with anti-TNA or anti-EPF antibodies (500 μg/mL) or TM60 (60 μg/mL) for 10 min at 22 °C prior to the binding experiments. An aliquot (15 μL) was then taken into a total assay volume of 50 μL, and binding of α-thrombin was measured as described above. Using ^{125}I-α-thrombin as ligand, binding data were determined with platelets from four normal donors in the presence and absence of anti-TNA and from four additional donors only in the presence of anti-TNA antibodies. For ^{125}I-PPACK-thrombin, binding data were obtained using platelets from 3−5 different donors in the presence of anti-TNA and anti-EPF antibodies or using preimmune rabbit IgG as a negative control.

Data Analysis. Binding data were obtained for each of five experimental groups; namely, controls + α-thrombin ($n = 4$); controls + α-thrombin in the presence of anti-TNA ($n = 8$); controls + PPACK-thrombin ($n = 5$); controls + PPACK-thrombin in the presence of anti-TNA ($n = 3$); and controls + PPACK-thrombin in the presence of anti-EPF ($n = 3$). The data obtained from individual binding assays in each experimental group were coanalyzed by curve-fitting using the LIGAND program (Munson, 1983). The binding parameters obtained by this analysis were the apparent binding capacity of the saturable receptor (*R*) and the apparent equilibrium binding constant of dissociation or the free ligand concentration giving half-saturation for each of the binding sites (*K*). Nonspecific binding (NS), which denotes the ratio of nonspecifically bound to free thrombin, was treated as a parameter subject to error and was fit simultaneously with other parameters. Several models were examined to obtain the best description of the binding data. These included the following: (1) a model of a single site with nonspecific binding; (2) a model of two independent

binding sites without nonspecific binding; (3) a model of two independent binding sites with nonspecific binding; and (4) a model of three independent binding sites without nonspecific binding. Objective statistical criteria (*F*-test, extra sum of squares principle) were used to evaluate the goodness of fit and for discriminating between different models.

RESULTS

Peptide and Antibody Characterization. The DEG peptide (250 μM), the TNA peptide (200 μM), and the LLR peptide (200 μM) prolonged the thrombin clotting time of pooled human plasma to >400 s at 5 nM α-thrombin and completely inhibited the hydrolysis of S2238 and platelet aggregation induced by 0.5 nM α-thrombin. However, the EPF peptide (200 μM) was ineffective in any of these three assays, suggesting that the active sequence may be the overlapping domains of the TNA and LLR peptides, namely, LLRNP. The ability of the TNA and LLR peptides to prolong the thrombin clotting time is similar to observations on the interaction of related peptides with α-thrombin (Hung et al., 1992; Gralnick et al., 1994). We have found that preincubation of peptide $D^{269}-D^{287}$ (250 μM; numbering is based on the sequence of mature GPIbα) with α-thrombin (0.5 nM, 10 min, 37 °C) inhibited its proteolytic activity on the synthetic substrate S2238 as similarly reported for α-thrombin preincubation (0.3 nM, 5 min, 4 °C) with peptide $G^{268}-A^{291}$ (125 μM) (Katagiri et al., 1990). On the other hand, preincubation of α-thrombin (0.78 nM, 10 min, 22−25 °C) with 200 μM $D^{269}-E^{282}$ or $G^{271}-E^{286}$ has been reported not to inhibit S2238 proteolysis (De Marco et al., 1994). Apart from differences in procedure, the reason for these discrepancies is not known.

The polyclonal anti-TNA and anti-EPF peptide antisera had titers of $1 \times 10^4-5 \times 10^4$ at 3 × background optical density when measured against their respective antigens using an alkaline phosphatase ELISA assay. The total IgG fraction was isolated using ammonium sulfate-protein A and used for all subsequent experiments. Each antibody to the STDR peptides was specific for its peptide in ELISA assays, and neither showed any cross-reactivity with the DEG peptide corresponding to the thrombin binding domain of GPIbα (data not shown).

The monoclonal antibody TM60 gave a strong positive fluorescence with control platelets but not with Bernard-Soulier platelets which lack GPIb (Figure 1A,B). However, both control and Bernard-Soulier platelets gave identical positive reactions with anti-TNA (Figure 1C,D) and anti-EPF (Figure 1E,F) as well as with ATAP138, a monoclonal antibody against the STDR (Brass et al., 1992) (Figure 1G,H). Binding of antibodies to one receptor did not affect the binding of antibodies to the other receptor, showing that there was no cross-reactivity between the antibodies directed against GPIb and those directed against the STDR. In all experiments described below, negative controls of class-matched antibodies were used to rule out nonspecific effects.

Effect of Anti-STDR Antibodies on α-Thrombin Binding. Since preliminary studies showed no significant differences in the ability of anti-EPF peptide antibodies and anti-TNA peptide antibodies to inhibit the binding of α-thrombin to platelets, all further studies were carried out using anti-TNA. The binding isotherm from coanalysis of the data from four 21 triplicate-point experiments examining the binding of ^{125}I-

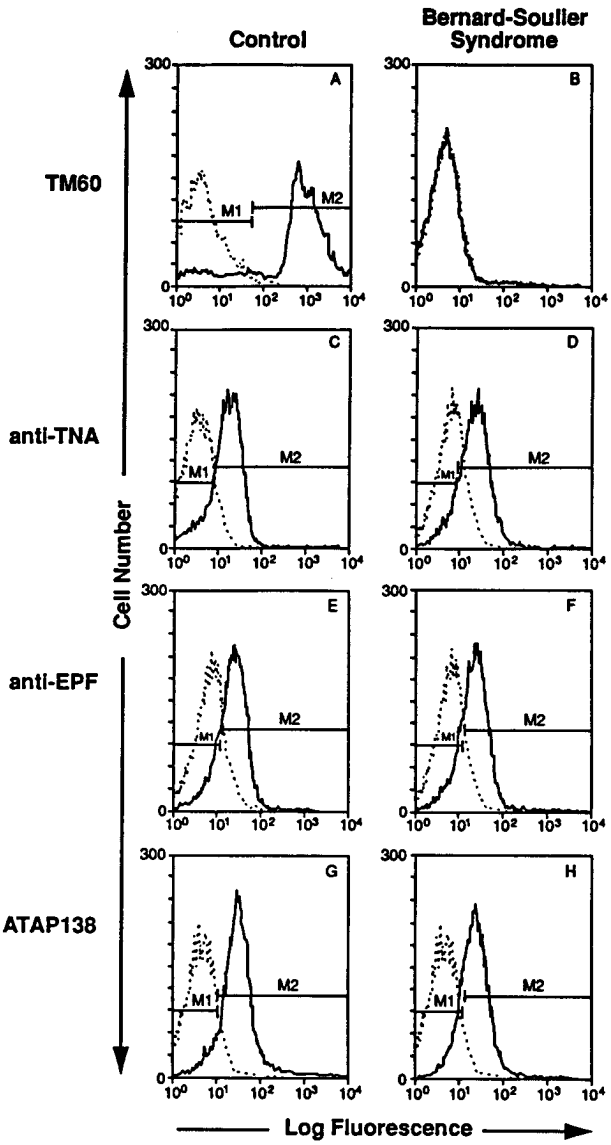

FIGURE 1: Antibody binding to control and Bernard-Soulier platelets. Intact, washed platelets were incubated with primary antibody in excess over the calculated amount of GPIb in control platelets (1 μg/mL). Bound antibody was then evaluated using a FITC-conjugated goat anti-species antibody and subsequent flow cytometric analysis (solid line; histogram marker M2). Nonspecific fluorescence (dot histogram) was measured using IgG_{2a} for TM60, preimmune serum for anti-TNA and anti-EPF, and IgG_1 for ATAP138 followed by the FITC-conjugated goat anti-species antibody (dotted line; histogram marker M1). Control platelets: panels A, C, E, G. Bernard-Soulier platelets: panels B, D, F, H. Reactions with TM60: panels A, B. With anti-TNA: panels C, D. With anti-EPF: panels E, F. With ATAP138: panels G, H.

α-thrombin to control platelets is shown in Figure 2 together with the corresponding binding isotherm generated from eight experiments (four of which were paired experiments with the controls) carried out in the presence of anti-TNA peptide antibodies. It is immediately apparent that the amount of thrombin bound to platelets (B/T ratio) is greatly reduced in the presence of the anti-STDR antibody. The data were examined for best fit to one-site, two-site, and three-site models with and without nonspecific binding. The best fit was for a two-site model with nonspecific binding as compared with one-site and three-site models. Each data set was then examined for the best fit to a two-site model deleting successively the terms attributable to high- and moderate-affinity binding sites. The best fit for the two-site model was obtained by the deletion of the moderate-

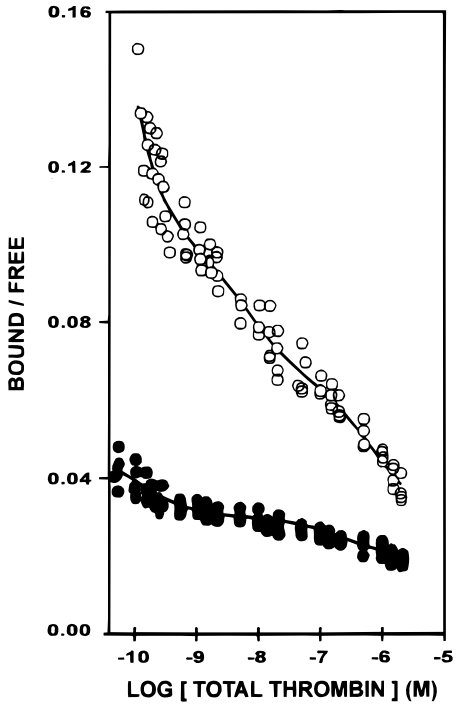

FIGURE 2: Effect of anti-TNA antibodies on α-thrombin binding to platelets. The points represent the experimental data of 21-point triplicate assays. The lines are the best fit to all the data points as determined by LIGAND analysis. Open circles are controls (n = 4), and filled circles are in the presence of anti-TNA (500 μg/mL) (n = 8). Control binding parameters were not affected by preimmune IgG (500 μg/mL).

affinity component. Statistical data for all models are given in Table 1. The data from these binding experiments were then coanalyzed, and a single binding isotherm was generated from all the data points and used to calculate the binding parameters.

The binding constants are summarized in Table 2. For controls, the binding constants at the high-affinity site (K_{d_1} 0.60 ± 0.17 nM), at the moderate-affinity site (K_{d_2} 25 ± 7 nM), and the low-affinity site (K_{d_3} 1450 ± 500 nM) are in good agreement with those reported from similar binding studies subjected to LIGAND analysis (Harmon & Jamieson, 1985, 1986, 1988; De Marco et al., 1991; Harmon et al., 1986) as are the number of sites per platelet for the high-, moderate-, and low-affinity sites, respectively 66 ± 20, 2800 ± 1600, and 87 800 ± 24 000. The number of binding sites and affinities for ^{125}I-thrombin at these sites were not affected by incubation with preimmune IgG (500 μg/mL). In the presence of anti-TNA peptide antibodies, there was no detectable binding of α-thrombin binding to the moderate-affinity site in the best-fit model. At the high-affinity site, the number of sites per platelet was decreased to 10 ± 5 while the affinity was increased to 0.10 ± 0.04 nM. At the low-affinity site, the number of sites was reduced to 4700 ± 1100 while the affinity was increased to 210 ± 50 nM. Nonspecific binding was not significantly different between controls and platelets blocked with anti-TNA.

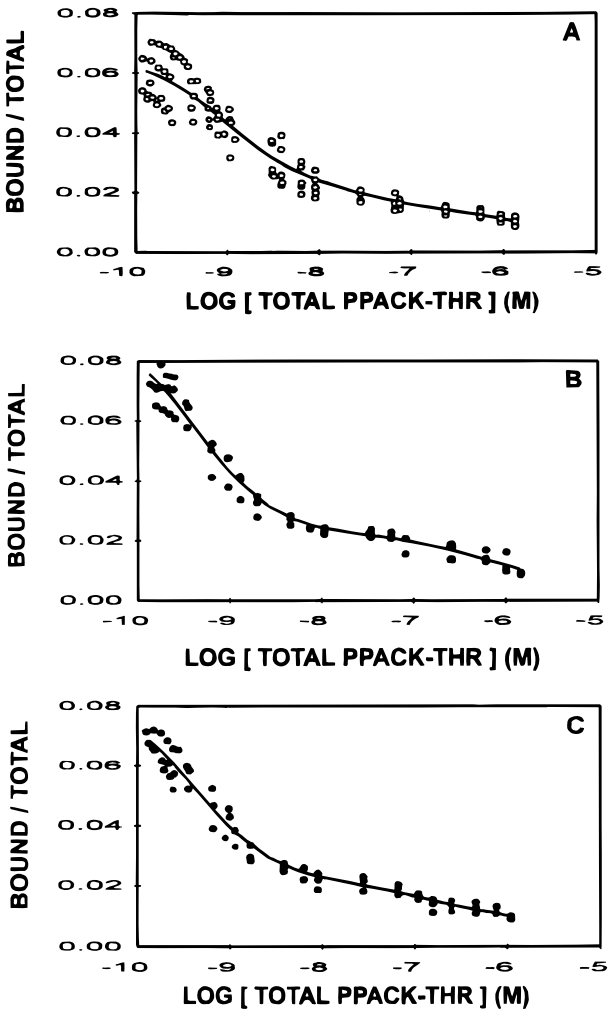

FIGURE 3: Effect of anti-STDR antibodies on the binding of ^{125}I-PPACK-thrombin to platelets. Conditions are as described in the caption of Figure 2. (A) Binding of ^{125}I-PPACK-thrombin to platelets in the presence of increasing concentrations of unlabeled PPACK-thrombin. (B) Binding of ^{125}I-PPACK-thrombin in the presence of anti-TNA antibodies (n = 3) (500 μg/mL). (C) Binding of ^{125}I-PPACK-thrombin in the presence of anti-EPF antibodies (n = 3) (500 μg/mL).

In order to avoid possible effects on the B/T ratio that may have arisen from the presence of active α-thrombin, further binding studies were carried out using thrombin blocked at its active site, namely, PPACK-thrombin (Figure 3). PPACK-thrombin binds similarly to α-thrombin in intact

Table 1: Parameters for Best-Fit Model[a]

	one site				two site				three site			
	sum of squares	Df	F	p	sum of squares	Df	F	p	sum of squares	Df	F	p
ligand: α-thrombin												
control	12942	75	69	0.001	3971	76	12.4	0.001	3406	75	- - -	- - -
plus anti-TNA	12891	135	93.5	0.00l	5357	133	- - -	- - -	**	**	**	**
plus anti-TM60	3308	38	2.8	0.001	1271	36	- - -	- - -	**	**	**	**
ligand: PPACK-thrombin												
control	20681	98	25.7	0.001	13470	96	2.74	0.01	13098	95	- - -	- - -
plus anti-TNA	13411	58	58.4	0.001	4344	56	- - -	- - -	**	**	**	**
plus anti-EPF	14822	58	75	0.001	3999	56	- - -	- - -	42331	63	0.46	0.859

[a] Df, degrees of freedom; F, F ratio test; p, level of significance. (- - -) Best-fit model; (**) program suggested an unfit model.

Table 2: Thrombin Binding Data[a]

	high affinity		moderate affinity		low affinity		
	K_d (nM)	sites/plt	K_d (nM)	sites/plt	K_d (nM)	sites/plt	NS
ligand: α-thrombin							
control ($n = 4$)	0.60 ± 0.17	66 ± 20	25 ± 7	2800 ± 1570	1450 ± 500	87760 ± 24060	$(4.08 \pm 2.45) \times 10^{-2}$
plus anti-TNA ($n = 8$)	0.10 ± 0.04	10 ± 5	nd	nd	210 ± 43	4720 ± 1120	$(1.93 \pm 0.58) \times 10^{-2}$
plus TM60 ($n = 2$)	nd	nd	3.3	190	260	23900	$(2.5 \pm 1.75) \times 10^{-2}$
ligand: PPACK-thrombin							
control ($n = 5$)	0.83 ± 0.29	80 ± 30	30 ± 21	502 ± 340	2400 ± 1600	59700 ± 29200	$(2.3 \pm 0.73) \times 10^{-3}$
plus anti-TNA ($n = 3$)	0.40 ± 0.17	60 ± 33	nd	nd	471 ± 25	14600 ± 9800	$(6.98 \pm 2.87) \times 10^{-3}$
plus anti-EPF ($n = 3$)	0.38 ± 0.19	53 ± 26	nd	nd	149 ± 98	3700 ± 3000	$(9.14 \pm 8.22) \times 10^{-3}$

[a] Values given are means ± SD. nd = not detectable in the best-fit model. NS = nonspecific binding or the ratio of nonspecifically bound to free ligand.

platelets (Harmon & Jamieson, 1986, 1988) (Figure 3A), and this was confirmed in the independent binding studies using α-thrombin and PPACK-thrombin described in Table 2. Coanalysis by the LIGAND program of all the ^{125}I-PPACK-thrombin binding data showed that a two-site model was the best fit in the presence of anti-TNA (Figure 3B) and anti-EPF (Figure 3C) while the fit was less good for a one-site model or a three-site model (Table 1). The fit with anti-TNA and anti-EPF was also better on deletion of the moderate-affinity site than of the high-affinity site. Using PPACK-thrombin, high-affinity binding and the number of sites in the presence of anti-TNA (K_d 0.40 ± 0.17 nM; 60 ± 33 sites/platelet) and anti-EPF (K_d 0.38 ± 0.19 nM; 53 ± 26 sites/platelet) were not significantly different from controls (K_d 0.83 ± 0.29 nM, 80 ± 30 sites/platelet) (Table 2). Nonspecific binding of PPACK-thrombin to platelets was about one-tenth that of α-thrombin. Differences at the lowest affinity sites were not significant taking into account the wide individual variation seen with different donors: for example, in a series of 13 normal controls, the number of low-affinity binding sites ranged from 2300 to 91 500 sites/platelet, and affinities at these sites ranged from 100 nM to 6.7 μM.

We also examined the effect of the anti-GPIb MoAb TM60 on binding of α-thrombin to platelets to determine whether its effects were similar to those previously reported for the anti-GPIb MoAb LJ-Ib10 (De Marco et al., 1991). The best fit for the binding data in this case was a two-site model of moderate- and low-affinity sites with no contribution from the high-affinity binding site. Moreover, at both moderate- and low-affinity sites, we found that affinity was increased and the number of available sites decreased in the presence of TM60 (Table 2).

Peak [Ca^{2+}] Values. Increasing concentrations of TM60, anti-TNA, or LJ-1b10 resulted in progressive decreases in peak $[Ca^{2+}]_i$ values induced by 0.5 nM α-thrombin, and a maximum inhibition of 50% of control values was reached at 120−150 μg/mL in each case (Figure 4). A combination of TM60 (150 μg/mL) and anti-TNA (150 μg/mL) essentially abrogated the ability of α-thrombin (0.5 nM) to induce increases in cytoplasmic $[Ca^{2+}]_i$. Inhibitors of ADP (ATPαS) and thromboxane production (aspirin) had no effect on thrombin-induced changes in $[Ca^{2+}]_i$ in the presence of these antibodies.

Peak $[Ca^{2+}]_i$ values in resting platelets from eight different donors were 97 ± 23 nM. With increasing concentrations of α-thrombin, there was a curvilinear increase in peak $[Ca^{2+}]_i$ which showed the greatest sensitivity to α-thrombin at concentrations below 2 nM and reached a value of 722 ± 88 nM at 10 nM α-thrombin (Figure 5). In the presence of the anti-TNA antibody against the STDR or of TM60 against

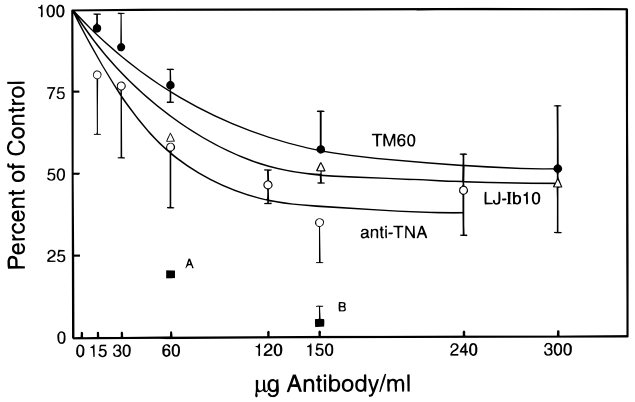

FIGURE 4: Inhibition of thrombin-induced peak $[Ca^{2+}]_i$ by anti-receptor antibodies. Results are given as mean ± standard deviation ($n = 3-10$ individual experiments using different donors). Data are presented as the percent of control values using 0.5 nM α-thrombin ($[Ca^{2+}]_i = 205 \pm 35$ nM) after subtraction of basal values in paired experiments. Point A is the $[Ca^{2+}]_i$ value in the presence of 60 μg/mL TM60 and 150 μg/mL anti-TNA ($n = 1$) while point B is the value in the presence of 150 μg/mL of each antibody ($n = 3$).

GPIbα, peak $[Ca^{2+}]_i$ values were half of those seen in control platelets over the whole range of α-thrombin concentrations examined. These differences in Ca^{2+} response between control platelets and antibody-blocked platelets were limited to activation with α-thrombin, and no differences were seen when the two platelet preparations were challenged with collagen (2.5 μg/mL), ADP (10 μM), arachidonate (100 μM), or ionomycin (1 μM) (data not shown).

Basal $[Ca^{2+}]_i$ values in Bernard-Soulier platelets (70 ± 24 nM) were similar to those of normal controls, and they showed normal increments in $[Ca^{2+}]_i$ of 180 and 150 nM, respectively, when challenged with U46619 (20 μM) and ionomycin (0.8 μM). In Bernard-Soulier platelets, peak $[Ca^{2+}]_i$ increased to only 154 ± 58 nM or ~20% of control platelets at 2 nM α-thrombin concentrations but increased rapidly to 50% of control values at higher α-thrombin concentrations where the response was indistinguishable from that of TM60-blocked platelets at 5 and 10 nM α-thrombin. The peak $[Ca^{2+}]_i$ response of 200 nM at 2 nM α-thrombin in Bernard-Soulier platelets was reduced to 145 nM at 500 μg/mL anti-TNA and to 90 nM at 1000 μg/mL, corresponding to 27% and 55% reductions, respectively: higher concentrations of anti-TNA were not examined.

Rate Effects. TM60 and anti-TNA not only reduced the *extent* of thrombin-induced increases in $[Ca^{2+}]_i$ but also affected the *rate* of increase, defined as the maximum $[Ca^{2+}]_i$ value reached divided by the time to reach that value. The rate of increase in control platelets was about twice that of antibody-blocked platelets and about 5-fold greater than the

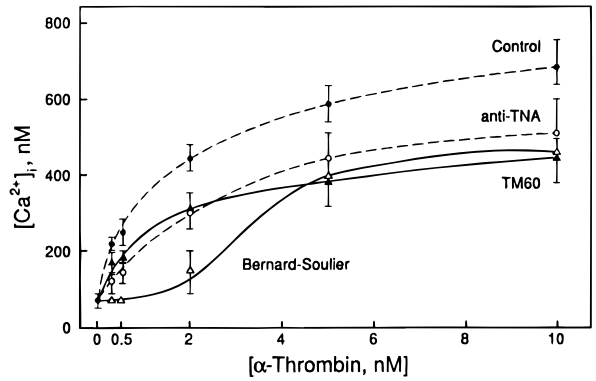

FIGURE 5: Concentration dependence of peak $[Ca^{2+}]_i$ responses. Platelets were incubated (10 min, 22 °C) without antibodies (control and Bernard-Soulier) or with TM60 (60 μg/mL) or anti-TNA (120 μg/mL) antibodies and were then challenged with increasing concentrations of α-thrombin as indicated. Peak $[Ca^{2+}]$ values are given as mean ± SD. Ca^{2+} values were determined with 4−6 different donors, and 2−4 determinations were carried out for each donor. Ca^{2+} determinations were carried out with the Bernard-Soulier donor on two different occasions. (●) Control platelets; (▲) TM60-blocked platelets; (△) Bernard-Soulier platelets; (○) anti-TNA-blocked platelets. Control and anti-TNA-blocked platelet Ca^{2+} responses are shown as dashed lines to improve clarity.

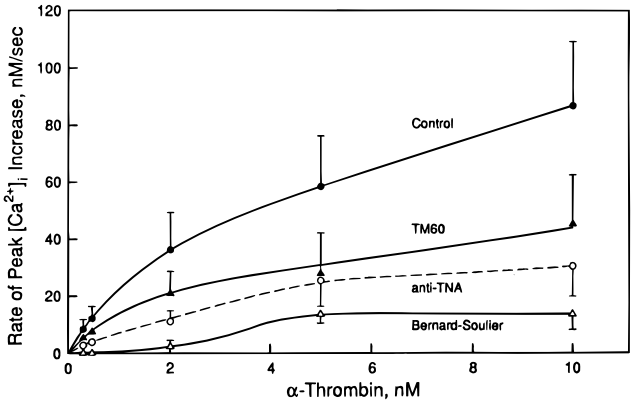

FIGURE 6: Rate of increase in $[Ca^{2+}]_i$. Rate is defined as the maximum $[Ca^{2+}]_i$ value divided by the time to reach that maximum. Ca^{2+} response curves summarized in Figure 5 were individually evaluated for maximum $[Ca^{2+}]_i$ values reached and for the time to reach that maximum in seconds.

peak rate of Bernard-Soulier platelets reached at 2 nM α-thrombin (Figure 6). The difference between control and TM60-blocked platelets is significant ($p < 0.025$) at all thrombin concentrations examined except 0.3 nM. The differences between controls and platelets blocked with anti-TNA were significant ($p < 0.004$) at all thrombin concentrations. Differences between the effects of TM60 and anti-TNA were not significant. The difference in rate between controls and Bernard-Soulier platelets was significant ($p < 0.005$) at all thrombin concentrations.

Effect of TLP on Peak $[Ca^{2+}]_i$. When peak $[Ca^{2+}]_i$ values were plotted as a function of TLP concentration (Figure 7), it was seen that the dose-response was identical in controls and in TM60-blocked platelets ($r = 0.9$). To put it another way, the effect of TLP on Ca^{2+} mobilization in control platelets is identical with that of α-thrombin in platelet preparations lacking a high-affinity receptor function. With Bernard-Soulier platelets, the maximum Ca^{2+} response was 50−60% of controls at all TLP concentrations, but the rate of increase in $[Ca^{2+}]_i$ was equal to that of controls (not shown), unlike the case with α-thrombin. Anti-TNA antibodies did not affect platelet activation by TLP.

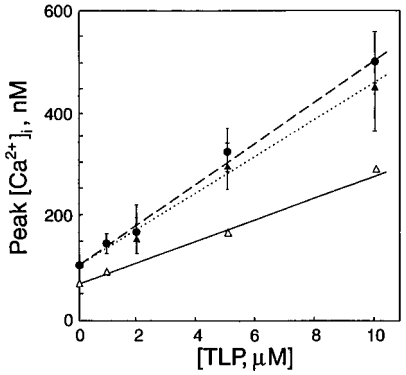

FIGURE 7: Concentration dependence of Ca^{2+} response to TLP. Bernard-Soulier platelets (△), normal platelets (●, - - -), and normal platelets in the presence of TM60 (60 μg/mL) (△, ···) were challenged with increasing concentrations of TLP as indicated. Peak $[Ca^{2+}]_i$ values are given as mean ± SD ($n = 3$, except for Bernard-Soulier platelets which were examined on two separate occasions from the same donor). The control peptide LLRNPNDKYEPFC (Vu et al., 1991) (100 μM) had no effect on $[Ca^{2+}]_i$.

DISCUSSION

Curve-fitting analyses of the binding of α-thrombin to platelets using the LIGAND program have generally conformed to a three-site model with zero or very low (∼2%) nonspecific binding or to a two-site model in which nonspecific binding includes the lowest affinity site. It must be emphasized, however, that adequate characterization of a three-site model requires a minimum of 20−22 data points over a wide range of α-thrombin concentrations, typically 0.1−1000 nM (Munson, 1983), and these conditions have not always been met. A survey of eight published studies carried out under these conditions shows a range of values for the dissociation constants of 0.1−1 nM at the high-affinity site, 11−54 nM at the moderate-affinity site, and 740−2400 nM at the low-affinity site. In the present work, curve-fitting for the binding of either α-thrombin or PPACK-thrombin to control platelets conformed to a three-site model with binding parameters within these ranges, but in the presence of anti-STDR antibodies, the best fit was a two-site model in which neither ligand bound at the moderate-affinity site. While these results supported the concept that the STDR corresponded to the moderate-affinity α-thrombin binding site, it was apparent that α-thrombin, but not PPACK-thrombin, also caused changes in binding at the high-affinity site.

It has been previously suggested (Brass et al., 1992) that the STDR may constitute the moderate-affinity thrombin receptor: (i) the STDR-derived peptide FLLRN inhibited moderate-affinity α-thrombin binding but did not affect high-affinity binding (Gralnick et al., 1994); and (ii) the number of platelet binding sites for the anti-STDR MoAb ATAP138 (∼1800) is identical with the number of moderate-affinity binding sites for α-thrombin (Brass et al., 1992). This suggestion was supported by our binding studies and was further supported by our observations that Bernard-Soulier platelets, which retain their moderate-affinity thrombin binding sites (De Marco et al., 1991), interact with anti-STDR antibodies as well as do control platelets. Furthermore, the functional molecular weight of the moderate-affinity site as determined by radiation inactivation and target analysis [30 000 ± 9000 (Harmon & Jamieson, 1985)] is in reasonable agreement with the peptide molecular weight of the STDR as deduced from the cloned cDNA (44 000) (Vu et al., 1991).

Although anti-STDR antibodies did not affect the high-affinity binding of PPACK-thrombin, the number of high-affinity sites with α-thrombin was reduced by about 85% while the affinity at those sites was increased about 6-fold. The LJ-Ib10 MoAb against GPIb has been similarly shown to reduce the number of moderate-affinity binding sites and to increase their affinity in addition to blocking high-affinity binding of α-thrombin to platelets (De Marco et al., 1991), and we have confirmed these observations with the anti-GPIb MoAb TM60. These similar findings from two laboratories using four different antibodies are a strong indication of the validity of this phenomenon. These effects do not appear to be due solely to the presence of active α-thrombin since binding at high-, moderate-, and low-affinity sites has previously been shown to be similar using as ligands α-thrombin, PPACK-thrombin, or mixtures of the two (Harmon & Jamieson, 1985, 1986, 1988). The critical factor in obtaining reduced binding at the receptors in the present case appears to be the presence of both active α-thrombin and an anti-receptor antibody. The down-regulation or reduced expression of the GPIb/IX complex in the presence of α-thrombin as determined by flow cytometry using anti-GPIb antibodies (Hourdille et al., 1992; Michelson et al., 1991; Nurden et al., 1995; White et al., 1995) may be related to these observations. Further studies are required to determine whether these secondary effects may involve the FcγRII receptor and steric or cooperative interactions between these different sites.

The mechanism and significance of the observed changes in binding affinities have not been established. As previously noted, high-, moderate-, and low-affinity binding sites have been characterized in intact platelets and isolated membranes. In the absence of high-affinity binding, as in Bernard-Soulier platelets (De Marco et al., 1991) and in *Serratia* protease-treated platelets (Greco et al., 1995), affinity at the moderate-affinity site is found to be increased about 10-fold to a K_d of 2−3 nM, suggesting that negative cooperativity may exist between high- and moderate-affinity sites in normal platelets. Using a soluble radioreceptor assay, we have previously identified two sites with binding affinities of 0.36 and 55 nM on purified GPIb and of 0.38 and 46 nM on purified glycocalicin, which is in excellent agreement with values found for the high- and moderate-affinity binding sites of whole platelets (Harmon & Jamieson, 1986) although their relationship to the binding sites characterized by radiation inactivation has not been established. More recent binding studies using immobilized glycocalicin (De Marco et al., 1994) or an immobilized recombinant fragment of GPIb (Marchese et al., 1995) gave a single site in each case with dissociation constants of 46 and 18 nM, respectively, which is in the range of the moderate-affinity receptor, suggesting that immobilization may affect α-thrombin binding. Peptides based on the sequence of the α-thrombin binding domain of GPIbα have been found to block high-affinity binding of α-thrombin to platelets and, in some cases (De Marco et al., 1994) but not in others (Gralnick et al., 1994), to decrease binding and increase affinity at the moderate-affinity site. These observations may be a reflection of the complex kinetics of inhibition of α-thrombin binding by glycocalicin seen in early studies (Okumura et al., 1978).

There have been several reports on the effects of anti-GPIb and anti-STDR antibodies on platelet function, but the effects of a combination of antibodies against the two receptors have not been previously examined. We have found that TM60 and anti-TNA in combination completely inhibit thrombin-induced elevations in $[Ca^{2+}]_i$. In the presence of either anti-TNA or TM60 alone, α-thrombin induced curvilinear responses in $[Ca^{2+}]_i$ that were half of control values at α-thrombin concentrations up to 10 nM. It has been emphasized that the amount of active thrombin available for platelet activation at a site of injury may only be about 0.5 nM due to potent antithrombin mechanisms, particularly the binding of α-thrombin to thrombomodulin on the endothelial cell surface (De Marco et al., 1991). Even in shed blood having only humoral antithrombotic mechanisms, clotting and maximal release of PF-4 occur at α-thrombin concentrations of ∼2 nM (Shuman & Levine, 1978). It may be noted that the dose-response curve of control platelets shows that their greatest sensitivity to incremental increases in α-thrombin occurs in this concentration range. Furthermore, the rate of increase in $[Ca^{2+}]_i$, defined as the peak $[Ca^{2+}]_i$ value reached divided by the time to reach that peak, was markedly reduced when the access of thrombin to either receptor was blocked with the corresponding antibody. Bernard-Soulier platelets showed no detectable Ca^{2+} response at low concentrations of α-thrombin (0.3 nM, 0.5 nM) and only about 50% response at higher concentrations (5 nM, 10 nM) which were indistinguishable from the values seen in platelets blocked with TM60 or anti-STDR antibodies while the rate of increase in $[Ca^{2+}]_i$ was even less. These results show that the absence of the high-affinity binding site has profound effects on both the extent and the rate of Ca^{2+} response induced by α-thrombin even in the presence of a fully active STDR.

The results of the present studies are in agreement with continuous-flow kinetic studies of rapid $[Ca^{2+}]_i$ dynamics in platelets activated by α-thrombin and the analogues, γ- and diisopropyl fluorophosphate-treated α-thrombin (Jones et al., 1989). These studies demonstrated that although the high-affinity binding activity of thrombin is not required for a major increase in cytoplasmic Ca^{2+} concentration, fully active α-thrombin acting at both sites induces a more rapid (≤2 s) mobilization of Ca^{2+}. It was further suggested that the earliest Ca^{2+} mobilization in response to ADP and α-thrombin involves a common mechanism, different from phospholipase C and IP_3 (Jones & Gear, 1988). Taken together, these results suggest that whereas the STDR pathway acts by means of phospholipase C and IP_3 to mobilize large stores of Ca^{2+}, a more rapid mobilization is evoked by the combined responses of the GPIb complex and STDR pathways.

Based on published results from several laboratories, we have previously proposed a two-receptor model to explain thrombin-induced platelet activation (Jamieson, 1988; Greco & Jamieson, 1991). The basic hypothesis proposed in this model is that GPIb is the high-affinity thrombin receptor and is coupled to platelet activation through phospholipase A_2 while the STDR is the moderate-affinity thrombin receptor and is coupled to platelet activation through phospholipase C. The following recent observations are in accord with this hypothesis: (a) the STDR has been shown to activate phospholipase C (Huang et al., 1991); (b) the 14-3-3 signal transduction protein which may have phospholipase A_2 activity has been shown to be associated with the GPIb/IX complex (Du et al., 1994); and (c) phospholipase A_2 itself has been shown to be activated by the binding of vWF to GPIb (Chow et al., 1992). These studies, together with our present work, confirm the essential correctness of the

proposed two-receptor model for the activation of platelets by α-thrombin. In another model, based on only 10-point binding assays, the high-affinity binding site on GPIb has been proposed as not being a receptor coupled to activation but as functioning to sequester small amounts of thrombin on the platelet surface and thereby preventing platelet activation through the STDR (Hayes et al., 1994; Leong et al., 1992). This model would imply that Bernard-Soulier platelets, which cannot sequester thrombin in this manner, should be more sensitive to α-thrombin than control platelets whereas the reverse is known to be the case, by an order of magnitude.

In summary, our results demonstrate that both GPIb and the STDR are required to ensure the maximal rate and extent of thrombin-induced increases in $[Ca^{2+}]_i$ during platelet activation and that the STDR corresponds to the previously-described moderate-affinity platelet receptor.

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